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Yeast Rrp9p is an evolutionarily conserved U3 snoRNP protein essential for early pre-rRNA processing cleavages and requires box C for its association

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ABSTRACT

Pre-rRNA processing in eukaryotic cells requires participation of several snoRNPs. These include the highly conserved and abundant U3 snoRNP, which is essential for synthesis of 18S rRNA. Here we report the characterization of Rrp9p, a novel yeast U3 protein, identified via its homology to the human U3-55k protein. Epitope-tagged Rrp9p specifically precipitates U3 snoRNA, but Rrp9p is not required for the stable accumulation of this snoRNA. Genetic depletion of Rrp9p inhibits the early cleavages of the primary pre-rRNA transcript at A_0, A_1, and A_2 and, consequently, production of 18S, but not 25S and 5.8S, rRNA. The hU3-55k protein can partially complement a yeast *rrp9* null mutant, indicating that the function of this protein has been conserved. Immunoprecipitation of extracts from cells that coexpress epitope-tagged Rrp9p and various mutant forms of U3 snoRNA limits the region required for association of Rrp9p to the U3-specific box B/C motif. Box C is essential, whereas box B plays a supportive role.

Keywords: nucleolus; ribosome; rRNA processing; U3 snoRNA; WD40 repeat

INTRODUCTION

Formation of eukaryotic ribosomes largely takes place in a specialized nuclear compartment, the nucleolus. The molecular details of ribosome biogenesis have been studied most extensively in the yeast *Saccharomyces cerevisiae* (Raué & Planta, 1991; Woolford & Warner, 1991) but the basic outline of the process appears to be the same in all eukaryotes.

Three of the four eukaryotic rRNAs (18S, 5.8S, and 25S/28S rRNA) are synthesized as a single precursor that contains two external (5′- and 3′-ETS) and two internal (ITS1 and ITS2) transcribed spacers. Upon completion of transcription, the pre-rRNA is extensively modified, mainly by 2′-*O*-ribose methylation and pseudouridine formation (Ofengand & Fournier, 1998; Bachellerie et al., 2000). Subsequently, the transcribed spacers are removed in a complex series of processing steps, carried out by endo- and exoribonucleases. At the same time, ribosomal proteins are assembled on the successive processing intermediates in an ordered fashion to produce the ribosomal subunits (Raué & Planta, 1995; Venema & Tollervey, 1999).

Most, if not all, aspects of ribosome synthesis involve participation of small nucleolar ribonucleoproteins (snoRNPs; Tollervey & Kiss, 1997). To date about 70 snoRNPs have been identified in yeast (Samarsky & Fournier, 1999), and an even larger number are present in higher eukaryotic cells. SnoRNPs can be classified into four groups on the basis of in vivo function and the conserved sequence elements present in their RNA components. The two largest classes are the box C/D and box H/ACA snoRNPs that play a crucial role in 2′-*O*-methylation and pseudouridylation of pre-rRNA, respectively. These snoRNPs determine the substrate site for the actual modification enzyme via direct base pairing between a guide sequence in their snoRNA and a specific region in the pre-rRNA (Bachellerie et al., 2000). The third class encompasses snoRNPs that also contain either the box C/D or box H/ACA sequence elements, but are functionally distinct. Members of this class are the highly conserved U3 and U14 (Kass et al.,

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1990; Li et al., 1990; Savino & Gerbi, 1990; Hughes & Ares, 1991), the metazoan U8 and U22 (Peculis & Steitz, 1993; Tycowski et al., 1994), and the yeast snR10 and snR30 snoRNPs (Tollervey, 1987; Morrissey & Tollervey, 1993), all of which are directly involved in particular pre-rRNA processing reactions leading to the mature form of either 18S or 25/28S rRNA. Finally, RNase MRP is specifically required for a cleavage reaction in ITS1 leading to the synthesis of the major, short form of 5.8S rRNA (Chu et al., 1994; Lygerou et al., 1996) and forms a class by itself.

U3 is the most conserved and abundant snoRNP. It has been identified in all eukaryotic organisms examined so far, where it is involved in the initial processing of the primary pre-rRNA transcript (Kass & Sollner-Webb, 1990; Borovjagin & Gerbi, 1999). In yeast, U3 is essential for processing at the three early cleavage sites A_0, A_1, and A_2 (Fig. 7) leading to the production of the mature 18S rRNA (Hughes & Ares, 1991). These cleavage reactions depend upon base pairing between the 5′ end of U3 snoRNA and specific sequences in both the 5′-ETS of the pre-rRNA and the 5′ stem-loop structure in the mature 18S rRNA (Beltrame & Tollervey, 1995; Hughes, 1996; Sharma & Tollervey, 1999). Similar interactions between U3 snoRNA and pre-rRNA were found to be essential for 18S rRNA synthesis in *Trypanosoma brucei* (Hartshorne & Toyofuku, 1999).

Whereas the function of the U3 snoRNA is well characterized, information on the nature and, in particular, the role of the protein components of this snoRNP is still limited. Yeast U3 shares three proteins, Nop1p, Nop56p, and Nop58p, with the other members of the box C/D family. All three are essential for pre-rRNA processing and homologs of these proteins appear to be present in metazoan box C/D snoRNPs (Jansen et al., 1991; Gautier et al., 1997; Watkins et al., 1998; Wu et al., 1998; Lafontaine & Tollervey, 1999, 2000; Lyman et al., 1999).

In addition to these generic box C/D proteins, five U3-specific protein components, Sof1p, Mpp10p, Imp3p, Imp4p, and Lcp5p, have so far been identified in yeast (Jansen et al., 1993; Dunbar et al., 1997; Wiederkehr et al., 1998; Lee & Baserga, 1999). Genetic depletion of any of these proteins causes a pre-rRNA processing phenotype identical to that observed upon depletion of U3 snoRNA, although the steady-state level of the snoRNA is not affected. Nothing is known about the specific roles of these proteins with the exception of Mpp10p, for which it was shown that C-terminal truncation blocks the pre-rRNA cleavages at A_1 and A_2, but not A_0 (Lee & Baserga, 1997). Homologs of Mpp10p (Westendorf et al., 1998) and Sof1p (S. Granneman and W.J. van Venrooij, unpubl. data) have been identified in human cells.

Lübben et al. (1993) purified a U3 RNA–protein complex from CHO cells that contains three proteins having apparent molecular weights of 55, 50, and 15 kDa. Recently, the human homolog of the 55-kDa protein was cloned and characterized (Pluk et al., 1998). hU3-55k is a member of the family of WD-40 repeat proteins and is specifically associated with U3 in the nucleolus. Its role in pre-rRNA processing remains to be determined. Database analysis identified a putative yeast homolog, whose amino acid sequence shows 33% identity and 58% similarity to that of hU3-55k. Here we report the characterization of this protein, which we have designated Rrp9p.

RESULTS

RRP9 is an essential gene

We disrupted the cloned *RRP9* gene by replacing 60% of its coding sequence with the *TRP1* gene. Integration of the *rrp9::TRP1* construct at the *RRP9* genomic locus in the diploid strain RS453 followed by sporulation resulted in a 2:2 segregation for cell viability with viable spores being Trp^- (data not shown). This clearly indicates that *RRP9* is an essential gene.

To confirm this conclusion, the *RRP9* gene was placed under control of the *GAL10* promoter and the fusion gene was integrated at the genomic *RRP9* locus by transformation into the wild-type haploid strain YJV140. Figure 1 shows the comparative growth analysis of the resulting *GAL::rrp9* strain YJV323 and the YJV140 parent. No difference in growth rate could be detected in liquid synthetic galactose medium. Upon shifting the cultures to glucose-based medium, which blocks transcription of the *GAL::rrp9* gene, both strains initially continue to grow with a doubling time of 1.7 h. Whereas

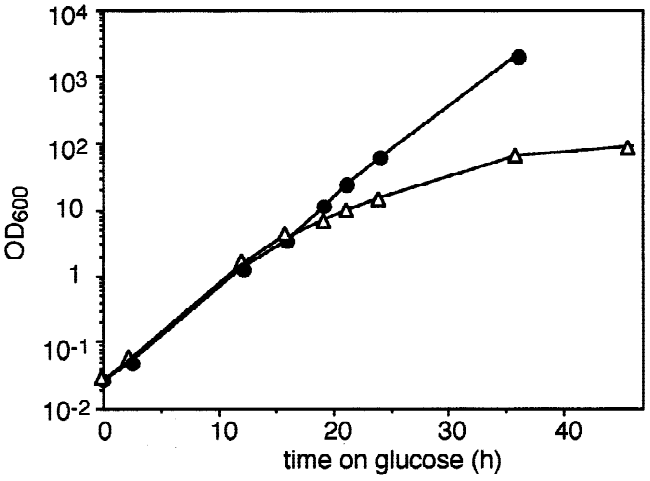

FIGURE 1. Rrp9p is an essential protein. Strain YJV323 containing a *GAL::rrp9* gene (△) as well as the wild-type isogenic *RRP9* control YJV140 (●) were grown on galactose-based medium and transferred to glucose-based medium at $t = 0$ h. Growth was followed by measuring the cell density. Cultures were periodically diluted to maintain exponential growth.

the wild-type *RRP9* strain maintains this growth rate over the duration of the experiment, the growth rate of the *GAL::rrp9* mutant starts to decline 12–15 h after the shift, and growth stops about 20 h later. This demonstrates that *RRP9* is indeed essential for cell viability.

RRP9p is a member of the family of WD40 repeat proteins

Previous sequence analysis of the hU3-55k protein (Pluk et al., 1998) showed the presence of five WD-40 repeats located between residues 142 and 352 (numbered I–V in Fig. 2A). Further analysis reveals a possible sixth copy (residues 370–404), which, however, lacks the consensus histidine residue at its start. Homologs of five of the WD-40 repeats are found in Rrp9p but their spacing differs from that in the human protein. In particular, large insertions are present in Rrp9p, relative to the human protein, between copies IV and V, as well as V and VI (Fig. 2A). Furthermore, the N-terminus of box A of copies III, V, and VI in Rrp9p does not contain the GH signature sequence (Fig. 2B).

Rrp9p is specifically associated with the U3 snoRNA

To identify the snoRNA(s) associated with Rrp9p the *RRP9* coding region was fused in-frame to a fragment encoding two IgG-binding domains of the *Staphylococcus aureus* Protein A, and the *ProtA::rrp9* gene was introduced on a low-copy number plasmid (pHIS3-ProtA::rrp9) into an *rrp9* null mutant by plasmid shuffling. Growth-rate analysis demonstrated that the tagged protein is fully functional (data not shown). Total lysates were prepared from yeast cells expressing either the tagged or the nontagged version of Rrp9p and incubated with IgG-agarose beads. After spinning down the beads, the supernatant and pellet fractions were analyzed by western blotting (Fig. 3A). Both fractions of the *ProtA::rrp9* extracts show a clear signal at the position expected for the fusion protein (molecular weight 79 kDa; Fig. 3A, lanes 3 and 4), whereas no signal is visible in the fractions from the control *RRP9* cells (Fig. 3A, lanes 1 and 2). Approximately 30% of the total amount of the ProtA-Rrp9p protein could be precipitated (Fig. 3A, compare lanes 3 and 4).

Total RNA was isolated from the supernatant and pellet fractions and analyzed by Northern hybridization using probes specific for U3, snR10, U14, and U24 (all box C/D snoRNAs), the box H/ACA snR8, and RNase MRP. As shown in Figure 3B, the pellet fraction of the *ProtA::rrp9* cells contained about 25% of the input U3 snoRNA, in good agreement with the percentage of Rrp9p that is precipitated. None of the other snoRNAs was found to be present in this fraction (Fig. 3C,D and data not shown).

To test whether association with Rrp9p is necessary for the stability of the U3 snoRNA, total RNA was isolated from the YJV323 (*GAL::rrp9*) and YJV140 (*RRP9*) strains at various times after the shift to glucose and analyzed by Northern hybridization using probes specific for U3 and snR10 snoRNA. Figure 4 makes it clear that the level of U3 snoRNA is only marginally reduced upon long-term depletion of Rrp9p. This strongly indicates that Rrp9p is a specific component of the U3 snoRNP. However, it is not required for the stable accumulation of U3 snoRNA.

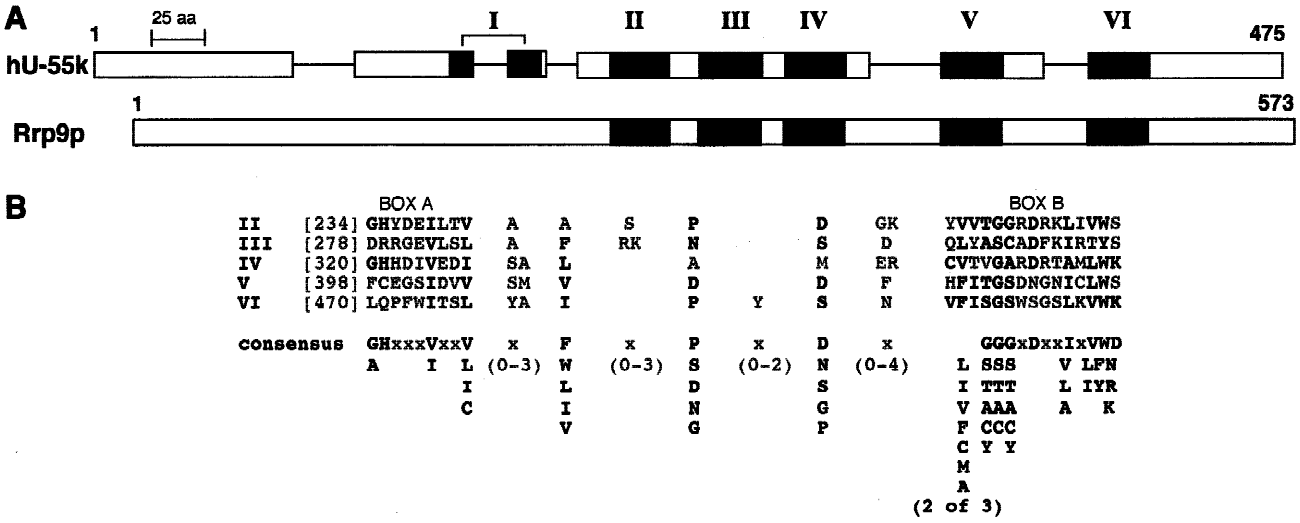

FIGURE 2. Structural comparison of the hU3-55k and Rrp9p proteins. **A**: Schematic alignment of the two proteins. Thin lines indicate gaps in the human sequence required for optimal alignment. WD-40 repeats are indicated by the black boxes. **B**: Sequence of WD-40 repeats II–VI of Rrp9p. The position of the residue at the N-terminus of each of the repeats is shown. Residues that satisfy the consensus are in bold.

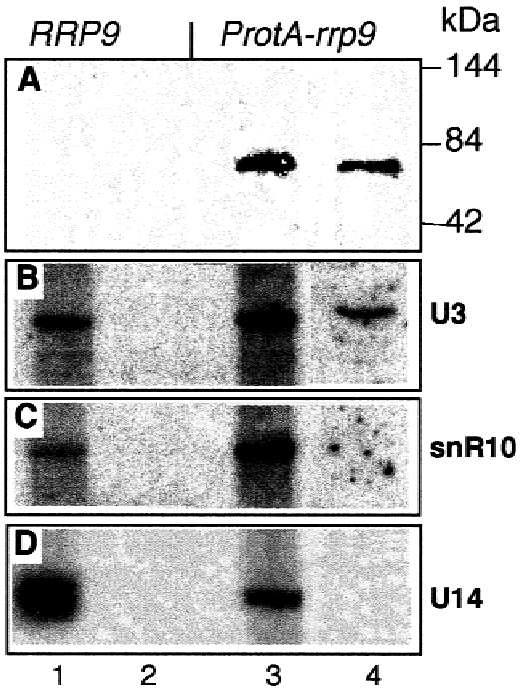

FIGURE 3. Rrp9p is a specific component of the U3 snoRNP. Whole-cell lysates were prepared from strains expressing Rrp9p (YJV447; lanes 1 and 2) or ProtA-Rrp9p (YJV448; lanes 3 and 4) and treated with IgG-agarose beads. Equivalent amounts of the supernatants (lanes 1 and 3) and the immunoprecipitates (lanes 2 and 4) were analyzed. **A**: Detection of ProtA-Rrp9p by western blotting using rabbit peroxidase antiperoxidase complex (PAP). The positions of molecular weight markers are indicated. **B–D**: Northern hybridization using probes detecting U3, snR10, and U14 snoRNA, respectively.

hU3-55k partially complements the *rrp9* null mutant

To ascertain that Rrp9p and hU3-55k are not only structural but also functional homologs, the cDNA encoding the hU3-55k cDNA was cloned into the single-copy pRS313 as well as the multicopy pRS423 vector under control of the *GAL10* promoter. The resulting pHIS3-GAL::hU3-55k plasmids were then transformed into YJV387 (*rrp9::TRP1* + pURA3-RRP9) and transformants were selected on SGal-his-trp. When these transformants were streaked onto SGal plates containing 5-FOA small, galactose-dependent colonies were obtained (data not shown). This indicates that the cells remain viable after losing the pURA3-RRP9 plasmid and, thus, that the hU3-55k protein can functionally replace Rrp9p in yeast.

To study the complementation in more detail both the single-copy (YJV514) and the multicopy (YJV513) transformant was plated in serial dilutions at four different temperatures (Fig. 5A). Comparison with the YJV387 control (*GAL::rrp9*) shows that complementation by the human gene is not complete. Although at 23 °C and 30 °C viability is restored, the cells still have an obvious growth defect. However, we consistently observed a somewhat higher growth rate for the multicopy transformant. Neither type of transformant grows at 16 °C and 37 °C.

These results were confirmed by measuring the growth rate in liquid SGal medium (Fig. 5B). At 30 °C the doubling time of YJV514 is approximately threefold higher than that of the *GAL::rrp9* control (6.4 h versus 2.1 h), whereas the YJV513 multicopy transformant has a doubling time of 4.2 h. Upon raising the temperature to 37 °C (arrow in Fig. 5B), growth of the *GAL::hU3-55k* cells gradually ceases, whereas the control cells continue to grow unimpeded. We conclude that the human hU3-55k and the yeast Rrp9p proteins are indeed orthologs.

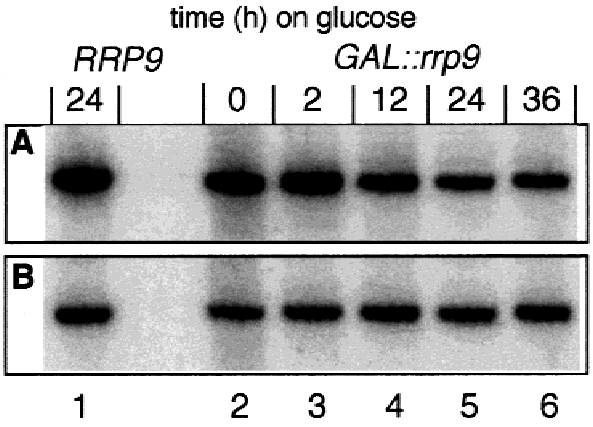

FIGURE 4. U3 snoRNA is stable upon depletion of Rrp9p. RNA was extracted from the *GAL::rrp9* strain YJV323 at different times after transfer to glucose (lanes 2–6) and analyzed by Northern hybridization using oligonucleotides detecting U3 (**A**) and snR10 (**B**) snoRNAs. As a control, RNA prepared from the *RRP9* strain YJV140 at 24 h after transfer to glucose was used (lane 1).

18S rRNA synthesis is blocked in the absence of Rrp9p and restored by *hU3-55k*

Total RNA was isolated from YJV323 (*GAL::rrp9*) and YJV140 (*RRP9*) at various times after the shift to glucose and the levels of the mature rRNAs were analyzed by Northern hybridization. As shown in Figure 6A, depletion of Rrp9p does not significantly affect the level of the 25S and 5.8S rRNAs. 18S rRNA remains constant until about 12 h after the transfer to glucose (Fig. 6A, lanes 2–4), consistent with the absence of any growth defect up to that time. At later times the level of 18S rRNA is severely reduced (Fig. 6A, lanes 5–6). We conclude that Rrp9p is specifically required for formation of 18S rRNA.

In agreement with the growth analysis data, introduction of the *hU3-55k* gene into the *rrp9* null mutant on a single-copy plasmid restores a low level of 18S rRNA production (Fig. 6B, lane 2). In cells transformed with the multicopy plasmid, the level of 18S rRNA is further increased as can be deduced from the higher 18S/25S signal ratio in these cells (Fig. 6B, lanes 3). This cor-

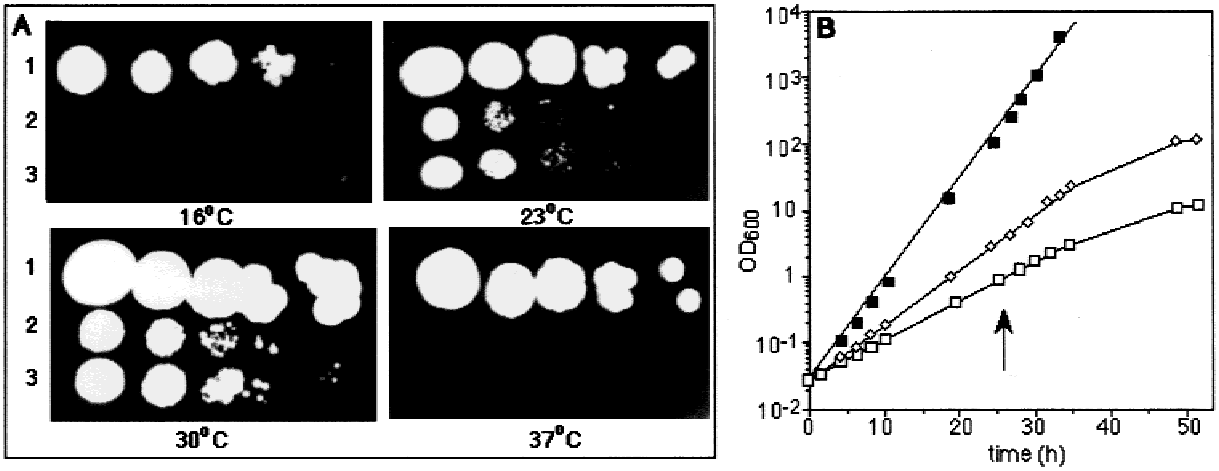

FIGURE 5. The human hU3-55k protein can partially complement an *rrp9* null strain. **A**: The *GAL::rrp9* control YJV387 (1), the single-copy *GAL::hU3-55k* YJV514 strain (2), and the multicopy *GAL::hU3-55k* YJV513 strain (3) were plated on galactose-based medium in 10-fold serial dilutions and incubated at four different temperatures. **B**: YJV514 (□) and YJV513 (◇) carrying a *GAL::hU3-55k* on a single-copy and a multicopy plasmid, respectively, as well as YJV387 (■) were grown in galactose-based medium. Cells were maintained at 30 °C from $t = 0$ to 26 h (arrow) and then shifted to 37 °C.

roborates our conclusion that the human and yeast proteins are true orthologs.

Rrp9p is required for processing at A_0, A_1, and A_2

To assess the role of Rrp9p in rRNA biogenesis in more detail we analyzed the levels of the various pre-rRNA processing intermediates in cells undergoing depletion of the protein. The yeast pre-rRNA processing pathway is depicted in Figure 7A and the locations of the oligonucleotide probes used to detect the different pre-rRNA species are shown in Figure 7B.

Depletion of Rrp9p causes a severe reduction in the levels of the 32S (Fig. 8 A), the $27SA_2$ (Fig. 8B), and the 20S (Fig. 8D) pre-rRNA species. As these pre-rRNAs result from cleavage at sites A_1 and A_2, we conclude that the absence of Rrp9p strongly inhibits these cleavage events. In all cases the time at which the level of the pre-rRNA species starts to decline (12 h after the shift) is in close agreement with the onset of the reduction in growth rate (Fig. 1).

Although probe #3 is also complementary to 33S pre-rRNA, the product of cleavage at site A_0 that would hybridize to probe #3 (Fig. 7), this precursor is notoriously difficult to detect by Northern hybridization. The effect of Rrp9p depletion on cleavage at this site was, therefore, analyzed by primer extension. As shown in Figure 8G, the primer extension signal corresponding to site A_0 starts to decrease strongly in intensity 12 h after the shift to glucose and has virtually disappeared 24 h after the shift. In agreement with these observations, depletion of Rrp9p leads to a strongly elevated level of the 35S primary transcript (Fig. 8A). Furthermore, it causes accumulation of the aberrant 23S pre-rRNA species (Fig. 8E), the product of direct processing of the 35S pre-rRNA at site A_3 within ITS1 (Tollervey, 1996). Processing at the latter site, as well as at sites B_1, is not impeded by Rrp9p depletion as demonstrated by the fact that the level of the 27SB pre-rRNA (Fig. 8C) remains constant up to 24 h after the shift. Similarly, processing in ITS2 is not affected, as can be concluded from the absence of any reduction in the level of the 7S pre-rRNA (Fig. 8F). Taken together, these results convincingly demonstrate that Rrp9p is specifically required for the processing cleavages at sites A_0, A_1, and A_2.

Box C of U3 snoRNA is essential for association of Rrp9p

Using an in vitro assay Lübben et al. (1993) were able to restrict the region of human U3 snoRNA required for stable association of the CHO 55-kDa protein to nucleotides 97–204, which correspond to the U3-specific boxes B and C as well as hairpins 2 and 3 of yeast U3 snoRNA (Fig. 9A). To identify the structural elements of yeast U3 involved in its association with Rrp9p, we introduced the *ProtA::rrp9* gene into yeast strain JH84 (*GAL::U3*) in combination with different, plasmid-encoded *U3* mutant genes containing a unique hybridization "tag" (kind gift of Dr. Samarsky; Samarsky & Fournier, 1998). The mutations tested include a deletion of the 5′-terminal region, which contains the sequences complementary to the pre-rRNA (Hughes, 1996; mutant ΔH), a deletion of hairpins 2–4 (mutant Δ2–4) and various sequence alterations in boxes B and C (see Fig. 9A).

Whole-cell extracts were prepared 4 h after a shift of the cultures from galactose-based to glucose-based medium to repress transcription of the genomic *GAL::U3* gene. Extracts were treated with IgG-agarose beads

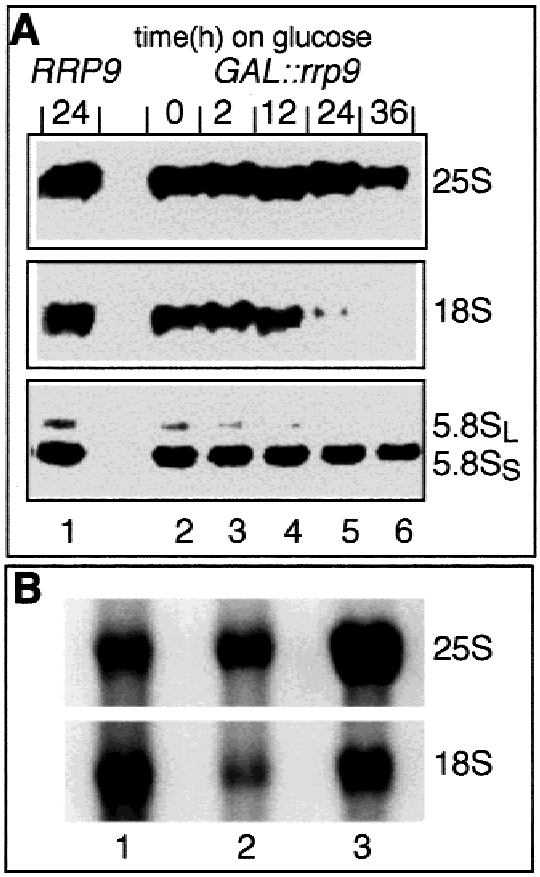

FIGURE 6. Synthesis of 18S rRNA specifically depends upon the presence of either Rrp9p or hU3-55k. **A**: Total RNA extracted from YJV323 (*GAL::rrp9*) at various times after transfer to glucose (lanes 2–6) was analyzed by Northern hybridization using oligonucleotides detecting mature 25S, 18S, and 5.8S rRNA, respectively (cf. Fig. 7B). Control RNA was prepared from YJV140 (*RRP9*) at 24 h after transfer to glucose (lane 1). **B**: Total RNA was extracted from YJV387 (*rrp9::TRP1* + pURA3-GAL::RRP9; lane 1), YJV514 (*rrp9::TRP1* + pHIS3-GAL::hU3-55k, low-copy; lane 2) and YJV513 (*rrp9::TRP1* + pHIS3-GAL::hU3-55k, high-copy; lane 3) separated by gel electrophoresis and the gel was stained with ethidium bromide.

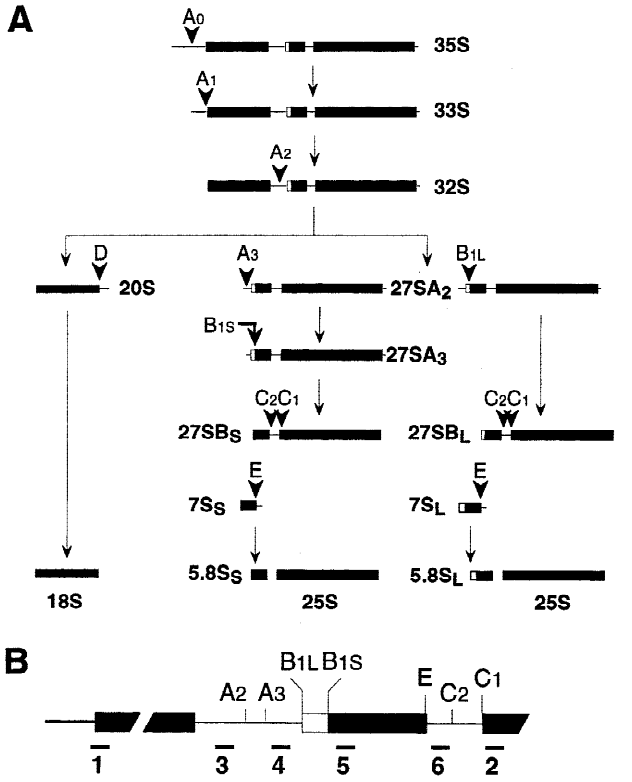

FIGURE 7. Processing of pre-rRNA in *Saccharomyces cerevisiae*. **A**: Schematic representation of the processing pathway. The mature 18S, 5.8S, and 25S rRNA sequences are shown as thick bars, transcribed spacer sequences as thin bars. Processing sites are indicated by arrows. **B**: Location of the oligonucleotide probes used in Northern hybridization and primer extension analysis.

and the material binding to the beads was analyzed by Northern hybridization. Mutant U3 species were detected with a probe complementary to the hybridization tag. The blot was then stripped and rehybridized with a probe specific for wild-type U3. Analysis of the original extracts showed that all mutant U3 species are expressed at about the same level (data not shown; see also Samarsky & Fournier, 1998).

Lane 1 of Figure 9B shows the background signals obtained with an extract prepared from cells expressing untagged Rrp9p. The specificity of the two probes is demonstrated in Figure 9B, lanes 2 and 3, representing extracts from *ProtA::rrp9* cells transformed with an untagged and a tagged, but otherwise wild-type, *U3* gene, respectively. Lanes 4–7 of Figure 9B show the analysis of the various mutations in boxes B and C. The most striking result is the absence of a signal for mutant U3 in lane 7, which indicates that full substitution of box C abolishes association with Rrp9p. In sharp contrast, a clear signal is obtained for U3 carrying a full substitution of box B (Fig. 9B, lane 6). However, the ratio of wild-type versus tagged U3 in the precipitate from the cells carrying the box B mutation (Fig. 9B, lane 7) is considerably higher than in the control cells (Fig. 9B, lane 2). Because in the original cell extracts this ratio did not significantly differ between mutant and control (data not shown; see also Samarsky & Fournier, 1998) this suggests that the box B substitution does have some negative effect on the association of Rrp9p. The same seems to hold for the two point mutations near the 5′-end of box C (Fig. 9B, lanes 4 and 5).

Analysis of two further mutants confirms the crucial role of box C in Rrp9p association: whereas the ΔH mutant form of U3, lacking the hinge region (Fig. 9A) can be coprecipitated efficiently with protA::Rrp9p (Fig. 9B, lane 8), coprecipitation is lost completely when the box C substitution is introduced into this mutant (Fig. 9B, lane 9).

To ascertain the role of hairpins 2, 3, and 4, we analyzed mutant Δ2–4, in which these hairpins have been deleted using a probe that spans box C′ and, thus, detects both the wild-type and the shorter, mutant U3 snoRNA. For comparison, mutant ΔH was also in-

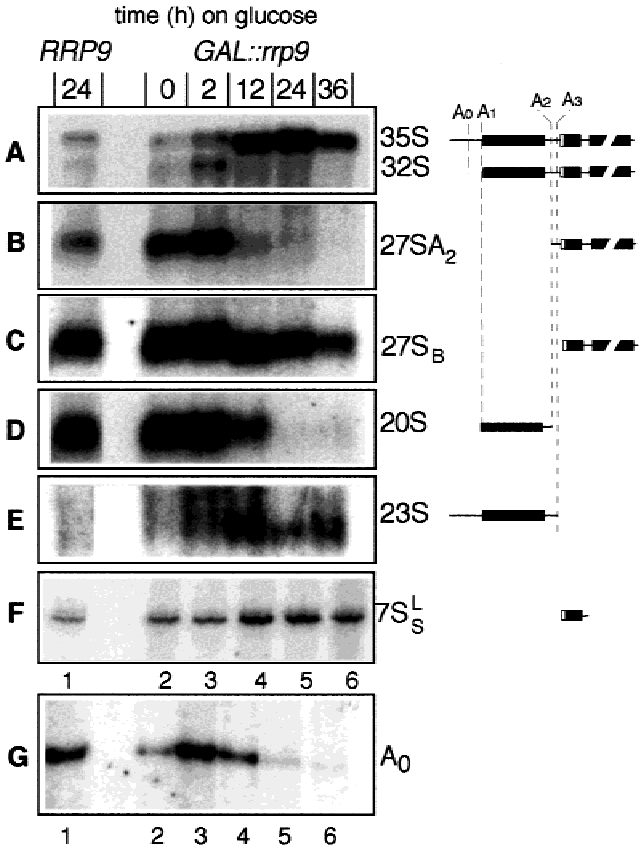

FIGURE 8. Depletion of Rrp9p inhibits the early cleavage steps in pre-rRNA processing. RNA was extracted from YJV140 control cells at 24 h (lane 1) and from YJV323 (*GAL::rrp9*) at various times after transfer to glucose (lanes 2–6) and analyzed by Northern hybridization (**A**–**F**) or primer extension (**G**). **A** through **F** represent consecutive hybridizations of the same filter with the different probes (cf. Fig. 7B). **A**, **D**, and **E**: Oligo #3. **B**: Oligo #4. **C** and **F**: Oligo #6. The structure of the various processing intermediates is indicated at the right. **G**: Oligo #1.

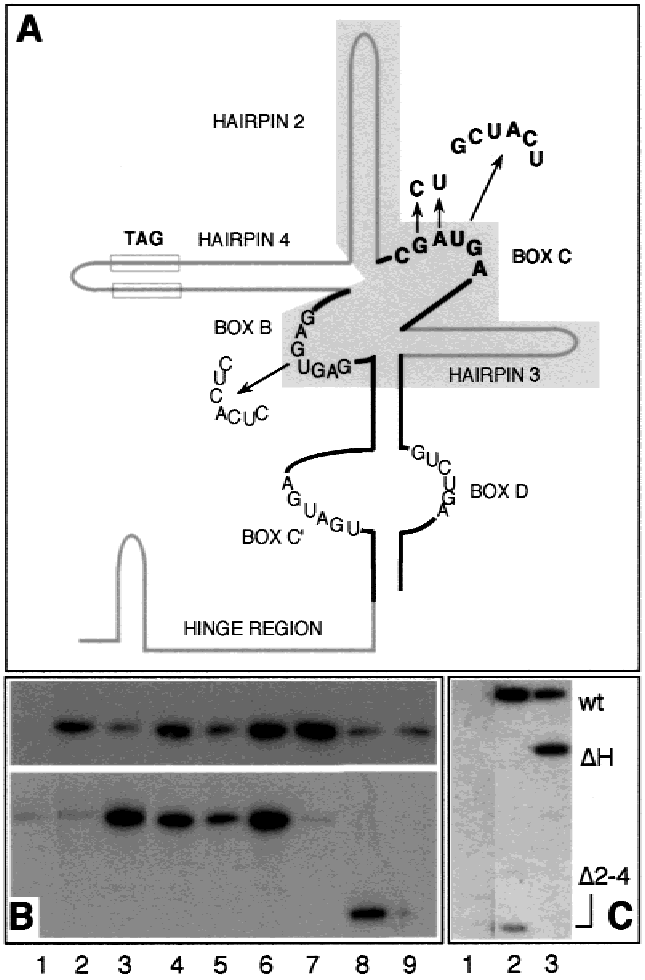

FIGURE 9. Box C is essential for the association of Rrp9p to U3 snoRNA. **A**: Schematic representation of yeast U3 snoRNA. Boxes in hairpin 4 represent the sequences inserted to create the unique hybridization tag (Samarsky & Fournier, 1998). Grey lines correspond to portions removed in the Δ2-4 and ΔH deletion mutants, respectively. Substitution mutations in boxes B and C are indicated. The portion of U3 corresponding to the region of human U3 snoRNA required for stable association of the CHO 55-kDa protein (Lübben et al., 1993) is shaded. **B**: Northern analysis of the pellet fractions after treatment with IgG agarose beads of extracts from cells co-expressing the ProtA-Rrp9p fusion protein and one of the mutant U3 snoRNAs. Upper panel: wild-type U3 detected by hybridization with a probe complementary to the wild-type sequence altered by insertion of the tag. Lower panel: hybridization of the same blot with a probe complementary to the tag present in the mutant U3 species. Lane 1: Control extract from *RRP9* cells. Lanes 2–10: extracts from *ProtA::rrp9* cells containing plasmid-encoded *U3* genes: lane 2: wild-type, untagged; lane 3: wild-type, tagged; lane 4: box C, Am → U; lane 5: box C, G → C; lane 6: box B substitution; lane 7: box C substitution; lane 8: ΔH; lane 9: ΔH + box C substitution. **C:** Northern analysis of pellet fractions of extracts from *RRP9* control cells (lane 1) and *ProtA::rrp9* cells containing the Δ2–4 (lane 2) and ΔH (lane 3) mutant *U3* genes, using a probe spanning the box C′ region.

cluded in this analysis. As shown in Figure 9C, clear signals corresponding to either mutant form of U3 are obtained. Thus, we conclude that the hairpins are not essential for association of Rrp9p. However, again a substantially higher ratio of wild-type/mutant U3 can be seen in the precipitate from the Δ2–4, compared to that from the ΔH, mutant (cf. Fig. 9B, lanes 2 and 3), suggesting that removal of the three hairpins also has a negative effect on this association.

From these data we conclude that box C is essential for the association of U3 snoRNA with Rrp9p whereas box B is of lesser importance but may still be involved. This conclusion is in agreement with the observation that the box B substitution does reduce the growth rate of cells expressing only this mutant form of U3 (Samarsky & Fournier, 1998). One or more of hairpins 2, 3, and 4 also seems to play a role. Similar results have been obtained for the association of the *Xenopus laevis* homolog of Rrp9p and its cognate U3 snoRNA (Lukowiak et al., 2000).

DISCUSSION

Using degenerate oligonucleotides based on peptide sequences of the purified U3 snoRNP-associated 55-kDa protein from CHO cells (Lübben et al., 1993), Pluk et al. (1998) cloned the cDNA encoding the human

homolog of this protein. A database search revealed the presence in yeast of two ORFs having significant sequence similarity to hU3-55k. One of these ORFs encoded Sof1p, which had already been identified as a U3-specific protein (Jansen et al., 1993). The other ORF, however, showed the highest degree of similarity and, therefore, was assumed to encode the actual homolog of the hU3-55k protein. Here we report a genetic and biochemical analysis of this protein, named Rrp9p. The results show that the yeast and human proteins are functionally equivalent and are essential for the early steps in pre-rRNA processing leading to production of 18S rRNA.

Coimmunoprecipitation experiments using a fully functional, epitope-tagged version of Rrp9p clearly indicate that Rrp9p is a specific component of the yeast U3 snoRNP. Whereas U3 snoRNA coprecipitates with epitope-tagged Rrp9p in approximately stoichiometric amounts, no other snoRNA, either of the C/D or the H/ACA class, can be detected in the immunoprecipitate (Fig. 3 and data not shown). This brings the total number of U3-specific protein components in yeast to six, including the previously identified proteins Sof1p, Imp3p, Imp4p, Mmp10p, and Lcp5p (Jansen et al., 1993; Dunbar et al., 1997; Wiederkehr et al., 1998; Lee & Baserga, 1999). Interestingly, all six U3-specific proteins, including Rrp9p, are essential for viability, but none of them is required for the stability of the U3 snoRNA.

Northern and primer extension analyses carried out on total RNA prepared from *GAL:rrp9* cells at various times after a shift from galactose-based to glucose-based medium traces the critical function of Rrp9p to the production of mature 18S rRNA (Fig. 6). Depletion of Rrp9p specifically blocks the early processing cleavages at A_0, A_1, and A_2 (Fig. 7) that are required for formation of 20S pre-rRNA, the immediate precursor of 18S rRNA. Cleavage of the primary 35S pre-rRNA transcript at A_3 and the subsequent processing of the $27SA_3$ precursor to 5.8S and 25S rRNA are not affected (Fig. 8). Again this phenotype is identical to that observed upon genetic depletion of any of the other U3-specific proteins (Jansen et al., 1993; Dunbar et al., 1997; Wiederkehr et al., 1998; Lee & Baserga, 1999) as well as upon loss of U3 snoRNA (Hughes & Ares, 1991). We are presently trying to determine whether the requirement for Rrp9p is a direct one or may be due to the fact that Rrp9p is necessary for the assembly of another, essential component into the U3 snoRNP.

Introduction of the gene encoding the hU3-55k protein into an *rrp9* null mutant restores 18S rRNA production and viability. However, the complementation is incomplete. Even at normal growth temperatures, yeast cells expressing the human protein show a significant growth defect due to underproduction of the 18S rRNA (Figs. 6 and 7). This indicates that either the human protein is inefficiently assembled into the yeast U3 snoRNP or yeast U3 snoRNP containing the hU3-55k protein is only partially functional. We consider the former explanation to be the most plausible one, based on two further observations. First, the human protein is unable to complement at either low (16 °C) or high (37 °C) temperature. Secondly, the growth rate and 18S rRNA production in *rrp9* null cells are further improved by increasing the expression level of the hU3-55k protein (Figs. 6 and 7). Both observations indicate that hU3-55k interacts less strongly with the yeast U3 components than its authentic yeast counterpart. Northern analysis of the RNA isolated from the *rrp9* null strains complemented with the *hU3-55k* gene demonstrated partial restoration of all three early cleavages (data not shown). Thus, it is clear that Rrp9p and hU3-55k are indeed orthologous proteins.

A notable feature of the hU3-55k protein is the presence of a total of six WD-40 repeats (Pluk et al., 1998; Fig. 2). Rrp9p also is an indisputable member of the family of WD-40 repeat proteins. However, there are substantial differences in the number, adherence to the consensus sequence, and spatial distribution of these repeats between the two proteins (Fig. 2). Interestingly, the U3-snoRNP contains a second WD-40 protein, namely Sof1p (Jansen et al., 1993). As WD-40 motifs appear to play a role in protein multimerization (Neer et al., 1994), direct interaction between Rrp9p and Sof1p could be an important architectural feature of the U3 snoRNP. The structural differences in the WD-40 repeat domain of hU3-55k might, therefore, be (partly) responsible for its less efficient association with yeast U3 snoRNP.

The (partial) complementation of an *rrp9* null mutant by the hU3-55k protein is only the second example of functional replacement of a snoRNP component by its counterpart from another organism. The first one was the functional replacement of the generic yeast box C/D snoRNP protein Nop1p by human fibrillarin, which incidentally also resulted in a temperature-sensitive phenotype (Jansen et al., 1991). However, possible orthologs of the remaining U3-specific proteins have been detected in various organisms either by biochemical analysis or by database searches (Jansen et al., 1993; Dunbar et al., 1997; Wiederkehr et al., 1998; Lee & Baserga, 1999; Lukowiak et al., 2000; S. Granneman and W.J. van Venrooij, unpubl. results). Similarly, Nop56p and Nop58p, the other two generic box C/D snoRNP proteins, have structural counterparts in other organisms (Gautier et al., 1997; Watkins et al., 1998; Wu et al., 1998; Lafontaine & Tollervey, 1999; Lyman et al., 1999). Thus, it seems likely that all of the U3-associated proteins have been conserved during evolution.

Lübben et al. (1993) showed that a region containing the U3-specific, conserved box B and box C sequence elements, but not boxes C′ and D common to all box C/D snoRNAs, is sufficient for stable association of the

CHO 55-kDa protein with human U3 snoRNA. Our coimmunoprecipitation experiments carried out on extracts from yeast cells that express the ProtA-Rrp9p fusion protein and different mutant forms of U3 snoRNA further limit the structural features of U3 that are essential for its association with Rrp9p to the U3-specific box C. A fully substituting mutation of box C abolishes coprecipitation in accordance with the inability of this mutant U3 to support growth (Samarsky & Fournier, 1998). The data further indicate that a similar full substitution of box B causes a much smaller reduction in the amount of mutant U3 in the immunoprecipitate. The same box B substitution was found to reduce the growth rate of cells dependent upon the mutant U3 snoRNA (Samarsky & Fournier, 1998). Thus, the conserved sequence of box B, although it appears to be important for fully efficient association with the Rrp9p protein, is not absolutely required. The G → C and A → U point mutants in box C also seem to have a negative effect on Rrp9p association, which, however, is considerably smaller than that of the full substitution. Thus, each of these 2 nt contributes to the association between U3 and Rrp9p. Finally, hairpins 2–4 may also contribute to the efficiency of Rrp9p association even though they constitute the least conserved structural feature of the U3 snoRNA. These hairpins could have a function in proper folding of the U3 snoRNA (Samarsky & Fournier, 1998), which in turn might be required for efficient association of the protein.

The negative effect of the box C point mutations and the Δ2–4 deletion on Rrp9p association seems to contradict the finding that these mutations do not affect the growth rate of cells that are fully dependent upon the mutant U3 snoRNA (Samarsky & Fournier, 1998). We can envisage at least two explanations for the absence of a deleterious effect of these mutations in vivo. First, the cellular level of U3 snoRNP may exceed the actual requirement for fully efficient pre-rRNA processing so that a limited reduction in the level of functional U3 would have no direct effect on the production of the mature rRNAs. Alternatively, the association of Rrp9p with the mutant U3 snoRNA may be more stable in vivo than under the conditions obtaining in the cell extract. A similar situation exists for some mutations in yeast ribosomal protein L25. Although these mutations lower the binding efficiency of the mutant protein to 25S rRNA in vitro, they have no effect on the cellular growth rate (Kooi et al., 1994).

U3 snoRNA lacking the 5′-terminal region (mutant ΔH) was found to be toxic in yeast cells when coexpressed with the wild-type form of U3. The toxic effect was abrogated by a fully substituting box C mutation, leading to the suggestion that the ΔH mutant U3 snoRNA competes with the wild-type form for a hypothetical *trans*-acting factor(s) (Samarsky & Fournier, 1998). Our data indicate that this hypothetical factor might be Rrp9p, which does associate with the 5′-terminally truncated U3 snoRNA, but requires an intact box C to do so (Fig. 9B).

The question of whether Rrp9p binds directly to the U3 snoRNA or associates via interaction with another protein component remains to be resolved. The fact that the metazoan homolog of Rrp9p appears to be a core component of the CHO U3 snoRNP (Lübben et al., 1993) would argue in support of direct binding. However, no known RNA-binding motif(s) can be distinguished in the sequence of either the hU3-55k protein or Rrp9p. Limited deletion analysis of the human protein demonstrated that the 17 C-terminal amino acids contain information that is essential for nucleolar localization and association with U3 snoRNA in vivo (Pluk et al., 1998). However, this sequence is not conserved between hU3-55k and Rrp9p, making it a poor candidate for direct interaction with box C.

Judged from its sedimentation coefficient in glycerol gradients, yeast U3 snoRNP contains approximately 10 proteins (Dunbar et al., 1997). Because the identification of Rrp9p brings the total number of U3 protein components, specific as well as generic, to nine, the inventory of yeast U3 proteins is at least nearly complete. Thus, U3 joins RNase MRP (Chamberlain et al., 1998) as the second snoRNP whose structural components are (almost) fully known. The next challenge will be to elucidate the architecture of the particle and the role of the various protein components in its assembly and biological function.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli strain MH1 was used for cloning and propagation of plasmids. Yeast transformation was carried out according to Gietz et al. (1992). Yeast strains used in this study are listed in Table 1. The pRS series of plasmids is described by Sikorski and Hieter (1989).

Cloning of the *RRP9* gene

A 2.8-kb region spanning the *RRP9* gene was amplified by PCR from total yeast DNA isolated from strain YJV140 (Table 1) using primers that introduced a flanking *Sac*I and *Kpn*I site. After cleavage with *Sac*I and *Kpn*I, the resulting fragment was cloned into pBS(KS+) and pRS316 (CEN-URA3), yielding pBS-RRP9 and pURA3-RRP9, respectively. pHIS3-RRP9 was constructed by cloning a 2.6-kb *Sac*I-*Xho*I subfragment into the corresponding sites of pRS313 (CEN-HIS3).

Construction of an *rrp9* null allele

A 1.25-kb *Sna*BI-*Nae*I fragment from pRS314 containing the *TRP1* gene was used to replace a 1.16-kb *Eco*RI-*Nco*I fragment of the *RRP9* gene of pBS-RRP9 (see above). A linear

TABLE 1. Yeast strains.

Strain	Genotype
RS453	*MAT***a**/*MATα ade2*/*ade2 his3*/*his3 leu2*/*leu2 trp1*/*trp1 ura3*/*ura3* (Sikorski & Hieter, 1989)
YJV140	*MAT***a** *ade2 his3 leu2 trp1 ura3*
YJV323	*MAT***a** *ade2 his3 leu2 trp1 ura3 GAL::rrp9 (URA3)*
YJV384	*MAT***a**/*MATα ade2*/*ade2 his3*/*his3 leu2*/*leu2 trp1*/*trp1 ura3*/*ura3 RRP9*/*rrp9::TRP1*
YJV386	*MAT***a** *ade2 his3 leu2 trp1 ura3 rrp9::TRP1* + pURA3-RRP9
YJV387	*MATα ade2 his3 leu2 trp1 ura3 rrp9::TRP1* + pURA3-RRP9
YJV447	*MATα ade2 his3 leu2 trp1 ura3 rrp9::TRP1* + pHIS3-RRP9
YJV448	*MATα ade2 his3 leu2 trp1 ura3 rrp9::TRP1* + pHIS3-ProtA::rrp9
YJV513	*MAT***a** *ade2 his3 leu2 trp1 ura3 rrp9::TRP1* + pHIS3-GAL::hU3-55k
YJV514	*MATα ade2 his3 leu2 trp1 ura3 rrp9::TRP1* + pHIS3-GAL::hU3-55k
JH84	*MATα leu2-3,12 ura3-52 his3-*Δ *ade2-1 can1100 u3a*Δ *GAL:U3A::URA3 U3B::LEU2* (Samarsky & Fournier, 1998)

2.47-kb *Nru*I-*Xho*I fragment containing the *rrp9::TRP1* allele was gel purified and transformed into the diploid strain RS453. YJV384 (Trp^+) was selected, transformed with plasmid pURA3-RRP9, and sporulated. Two plasmid-dependent haploid segregants, YJV386 and YJV387 (*rrp9::TRP1* + pURA3-RRP9), were selected.

Construction of the *GAL::rrp9* allele

A 1.5-kb blunt-ended *Bam*HI-*Hin*dIII fragment from pLGSD5 containing the *URA3* marker and the *GAL1/10* promoter cassette (Guarente et al., 1982) was cloned into the blunt-ended *Xba*I site of pBS-RRP9 located 12 bp upstream from the ATG start codon of *RRP9.* A purified 1.84-kb *Nru*I-*Eco*RI fragment from the resulting pBS-GAL::rrp9 was used to transform strain YJV140. Transformants were selected on SGal-ura plates and checked for galactose dependence on YPD and YPGal plates. The resulting *GAL::rrp9* strain was designated YJV323.

Construction of the *ProtA::rrp9* allele

A 400-bp blunt-ended *Nco*I fragment, encoding two IgG-binding domains of the *S. aureus* Protein A, was cloned into the blunt-ended *Xba*I site of pHIS3-RRP9, yielding pHIS3-ProtA::rrp9. This fuses the intact *RRP9* coding sequence in frame to the ProtA IgG-binding domain under control of the homologous *RRP9* promoter. Strain YJV387 was transformed with either pHIS3-ProtA::rrp9 or pHIS-RRP9. Transformants were selected on SD-trp-his plates and then streaked onto plates containing 5-FOA to perform a standard plasmid shuffle. Two 5-FOA-resistant strains, YJV447 (*rrp9::TRP1* + pHIS3-RRP9) and YJV448 (*rrp9::TRP1* + pHIS3-ProtA::rrp9) were selected.

Complementation by the human *U3-55k* gene

An *Nde*I and a *Bam*HI site were created by PCR at the start and stop codon, respectively, of the hU3-55k ORF present in pGEM3Zf-hU3-55k (Pluk et al., 1998). The *Nde*I-*Bam*HI fragment was cloned into PCR2.1 (Invitrogen) to give plasmid 1.3. The hU3-55k cDNA was fused to the *GAL10* promoter by cloning the blunt-ended *Nde*I-*Bam*HI fragment from plasmid 1.3 into the blunt-ended *Eco*RI site of pTL26 (CEN-HIS3) (Lafontaine & Tollervey, 1996). The resulting pHIS3-GAL::hU3-55k was transformed to YJV387 by selection on SGal-trp-his plates. Transformants were subjected to a plasmid shuffle on 5-FOA plates and YJV514 (*rrp9::TRP1* + pHIS3-GAL::hU3-55k) was selected for further analysis. Partial digestion of pHIS3-GAL::hU3-55k was used to obtain a 2.3-Kb *Eco*RI-*Xho*I fragment containing the *GAL::hU3-55k* DNA. This fragment was then cloned into the multicopy vector pRS423 and transformed into YJV386. After selection on SGal-trp-his and plasmid shuffle as described above, YJV513 was used for further analysis.

In vivo depletion of Rrp9p

YJV323 (*GAL::rrp9*) was grown in liquid SGal-ura medium until midexponential phase. Cells were harvested by centrifugation and resuspended to an OD_{600} of 0.06 in SD-ura. Cell growth was monitored over a period of up to 36 h, during which the cultures were regularly diluted with prewarmed medium to maintain exponential growth. As a control, YJV140 transformed with pRS316 (CEN-URA3) was used. For RNA isolation, cells were harvested at 0, 2, 12, 24, and 36 h after the shift to glucose-based medium.

RNA analysis

RNA isolation, Northern hybridization, and primer extension were carried out as described previously (Venema & Tollervey, 1996; Venema et al., 1998). For Northern hybridizations and primer extension, RNA samples corresponding to 0.4 and 0.04 OD_{600} units of cells were used, respectively. For primer extension a 10-fold molar excess of cold primer was mixed with the radiolabeled primer immediately before annealing. Oligonucleotides used for Northern hybridization are indicated in Figure 7.

Immunoprecipitation and western blot analysis

Cells were grown in YPD to midexponential phase, harvested by centrifugation, and washed once with ice-cold water. The pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 2 mM PMSF, 0.5% Triton X-100 (v/v)) at a concentration of 10 μL/OD_{600}-unit of cells. Cells were lysed by vortexing for 5 min with an equal volume of acid-washed glass beads (∅ 0.5 mm). A cleared lysate was prepared by two cycles of centrifugation for 5 min at 3000 × *g* and 4 °C and stored in aliquots at −80 °C. Immunoprecipitation was carried out as described previously (Bergès et al., 1994; Lygerou et al., 1994) using 20 μL lysate and 20 μL rabbit IgG-agarose beads (Sigma, St. Louis, Missouri). Pellet and supernatant fractions from the immunoprecipitation were separated on 8% SDS-PAGE gels

and blotted according to standard procedures. Western blots were decorated with rabbit peroxidase anti-peroxidase (PAP; Sigma) and developed using an ECL Plus detection kit (Amersham, UK). RNA was extracted from the material bound to the IgG-agarose beads by mixing the sample with an equal volume of 4 M GTC, extraction with phenol-chloroform and ethanol precipitation.

Association between ProtA:Rrp9p and various mutant forms of U3 snoRNA was analyzed in a similar manner using strain JH84 carrying pHIS3-ProtA::rrp9 as well as one of the sets of plasmids containing tagged, mutant forms of the *U3* gene (Samarsky & Fournier, 1998; kindly provided by Dr. Samarsky). After initial growth on galactose, cells were shifted to glucose-based medium to block transcription of the genomic *GAL-U3A* gene. Four hours after the shift whole-cells extracts were prepared and treated with IgG-agarose beads as described above. RNA was isolated from the supernatant and pellet fractions and analyzed by Northern analysis. Mutant U3 species were detected by using a probe complementary to the hybridization tag except in the case of the Δ2–4 mutant, where a probe complementary to the box C region was used. Specific detection of wild-type U3 was accomplished by using a probe complementary to the region whose sequence is altered by insertion of the tag (Fig. 9A).

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